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(71) Applicant : **SANYO CHEMICAL INDUSTRIES, LTD.**
11-1 Ikkyo Nomoto-cho
Higashiyama-ku Kyoto 605 (JP)

(72) Inventor : **Otagiri, Ken c/o Sanyo Chem. Ind. Ltd,**
11-1 Ikkyo Nomoto-cho Higasiyama-ku, Kyoto, 605 (JP)
Inventor : **Sugiura, Masakazu c/o Sanyo Chem. Ind.,Ltd,**
11-1, Ikkyo Nomoto-cho, Higasiyama-ku, Kyoto, 605 (JP)
Inventor : **Kouge, Katsushige**
777-3, Oaza Hirao-son, Hirao-cho
Kumage-gun, Yamaguchi-ken 742-11 (JP)
Inventor : **Koizumi, Tatsuya**
742-1, Oaza Hirao-son, Hirao-cho
Kumage-gun, Yamaguchi-ken 742-11 (JP)

(74) Representative : **Coleiro, Raymond et al**
MEWBURN ELLIS 2 Cursitor Street
London EC4A 1BQ (GB)

(54) Luminescent, luminometric assay and kits for use in the same.

(57) A chemiluminescent reaction, which may be utilized for immunoassay, carried out by reaction of 2,3-dihydro-1,4-phthalazinedione or its derivative, a peroxidase, and an oxidant, is enhanced by conducting it in the presence of a phenolic compound such as (4-cyanomethylthio)phenol. Especially, it is preferred that (4-cyanomethylthio)phenol is added to each of luminol, horseradish peroxidase, and a hydrogen peroxide to provide a significantly enhanced and stabilised luminescent reaction.

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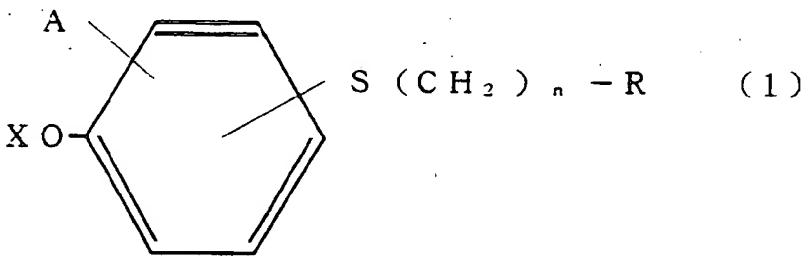
This invention relates to a luminescent and luminometric assay which rely upon reaction between 2,3-dihyro-1,4-phthalazine-dione or its derivative, a peroxidase enzyme, and an oxidant, in the presence of an enhancer. Furthermore, this invention relates to a diagnostic kit for use in the luminescent or luminometric assay comprising 2,3-dihydro-1,4-phthalazinedione or its derivative, a peroxidase, an oxidant, and the enhancer. Particularly the kit may utilize an immunoassay.

Gary H.G. Thorpe et al. disclose that a chemiluminescent reaction involving 2,3-dihydro-1,4-phthalazinedione or its derivative, a peroxidase enzyme, and an oxidant is carried out in the presence of a halogenated phenol such as paraiodophenol, and thus the chemiluminescent reaction is enhanced (Clinical Chemistry, vol.31, No. 8, p.1335)). Kricka et al, disclose methods of an assay or an immunoassay wherein the abovementioned luminescent reaction is carried out using a certain type of phenolic compound as an enhancer (US-A-4598044). Further in an assay of substances in biological fluids, there is the widely known enzyme immunoassay (EIA) wherein said substances or antigens of said substances are labelled by a peroxidase, and activity of the peroxidase is assayed by a colormetric assay involving an oxidant and (o-)phenylene-diamine which is a chromogen.

In the abovementioned chemiluminescent, if an enhancer could be found which provides more luminscence with less background and which can be used in smaller amounts is applicable in a luminmetric assay, it would be useful in that it would provide a wider range of application.

The present invention seeks to enhance the chemiluminescent reaction between a 2,3-phthalazinedione or its derivative, a peroxidase enzyme, and an oxidant. This allows an assay, especially an immunoassay, to provide an improved method of detecting and measuring 2,3-dihydro-1,4-phthalazinedione or its derivative, or a peroxidase enzyme by employing the abovementioned chemiluminescent reaction. Moreover, an assay kit for use in the abovementioned enhanced chemiluminescent may be particularly useful. According to an aspect of the present invention therefore, there is provided a chemiluminescent wherein the luminescent reaction is carried out involving a peroxidase enzyme, an oxidant, and a phenolic derivative of general formula(1):



(1)

wherein n represents an integer of 1 to 5; R represents hydrogen, cyano, morpholino, carboxylic acid, an alkoxycarbonyl group of 2 to 7 carbon atoms, metallic carboxylate, amido, aldehyde, or allyl, or is a phenyl group optionally substituted by a halogen atom; A is a hydrogen atom or a halogen atom; and X is hydrogen or alkaline metal. Preferably, depending upon the particular assay conditions, OX and $S(CH_2)_n$ -R are in the ortho or para position relative to one another.

According to another aspect, the invention provides an assay of peroxidase enzyme, 2,3-dihydro-1,4-phthalazinedione or its derivative involving the abovementioned chemiluminescent, especially an immunoassay involving this assay. Detection by the assay may be qualitative or quantitative, as desired.

According to a further aspect, the present invention provides an assay kit comprising a phenolic derivative of the general formula (1), an oxidant, and at least one of 2,3-dihydro-1,4-phthalazinedione or its derivative and a peroxidase.

In the chemiluminescent reaction of this invention which is enhanced by using the phenolic derivative of the general formula (1), light emission is, as compared with not using a phenolic derivative, significantly enhanced.

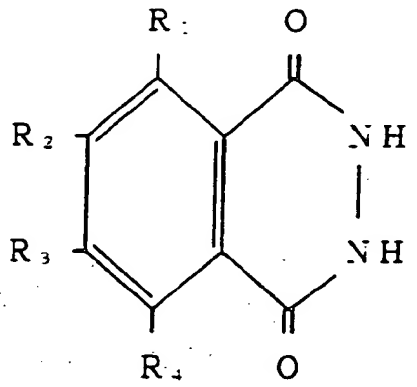
Specific examples of components used in this invention will now be described.

First, substances comprising (a) a phenolic derivative, (b) a 2,3-dihydro-1,4-phthalazinedione or its derivative, (c) a peroxidase enzyme and (d) an oxidant, which may be employed in the present invention will be described in detail.

The phenolic derivative (a) of a general formula (1), which is employed as an enhancer of a luminescent reaction is exemplified by the following: (4-benzylthio)phenol, (4-fluoro-benzylthio)phenol, (4-iodobenzylthio)

phenol, (4-chlorobenzylthio)phenol, (4-iodobenzylthio)phenol, (4-methylthio)-phenol, (4-cyanomethylthio)phenol, (4-cyanoethylthio)phenol, (4-cyanopropylthio)phenol, (4-cyanobutylthio)phenol, (4-cyanopentylthio)phenol, (4-morpholinomethylthio)phenol, (4-aminomethylthio)phenol, (4-nitromethylthio)phenol, (4-hydroxyphenylthio)-acetic acid, (4 hydroxyphenylthio)acetaldehyde, (4-hydroxyphenylthio)acetamide, (4-allylthio)phenol, 4-cyanomethylthio-2-fluorophenol, 4-cyanomethylthio-2-chlorophenol, 4-cyanomethylthio-2-bromophenol, 4-cyanomethylthio-2-iodophenol, and the sodium and potassium salts of any of these. Among these, preferred are (4-benzylthio)phenol, (4-methylthio)phenol, (4-cyanomethylthio)phenol, (4-morpholinomethylthio)phenol, 4-cyanomethylthio-2-fluorophenol, 4-cyanomethylthio-2-chlorophenol, 4-cyanomethylthio-2-bromophenol, and 4-cyanomethylthio-2-iodophenol, and the sodium salt and potassium salts of any of these. Most preferred are (4-cyanomethylthio)phenol, 4-cyanomethylthio-2-fluorophenol, 4-cyanomethylthio-2-chlorophenol, 4-cyanomethylthio-2-bromophenol, and the sodium and potassium salts of any of these.

The 2,3-dihydro-1,4-phthalazinedione or its derivative (b) which is used in this invention may be of the general formula (2):



(2)

wherein R_1, R_2, R_3 and R_4 are respectively a hydrogen atom, optionally unsubstituted or substituted C_1 to C_6 alkyl, optionally unsubstituted or substituted C_1 to C_6 alkenyl, hydroxyl, C_1 to C_6 alkoxy, carboxyl, amino or substituted amino group. Preferably, depending upon the particular assay conditions, at least one of R_1 or R_2 is an amino, or substituted amino (which includes amido), group.

Among the above compounds, preferred are luminol, isoluminol, N-aminohexyl-N-ethylisoluminol(abbr - viated to AHEI hereinafter), and N-aminobutyl-N-ethylisoluminol (abbreviated to ABEI hereinafter). The most preferred is luminol.

The 2,3-dihydro-1.4-phthalazinedione or its derivative (b) which is used in the assay of this invention may be used in a form, depending on a type of assay, coupled to a ligand such as an antigen, antibody, hapten, protein, avidin, and biotin, or in a free form. In the former case preferred ligands are antigens and antibodies. The ligand may be coupled directly to the 2,3-dihydro-1,4-phthalazinedione or its derivative (b) or via a coupling agent. As coupling agent, for example, conventional N-(m-maleimidobenzoyloxy)succunimide (MBS) may be employed. (T. Kitagawa et al : J. Bio. Chem., vol.79 p. 233 ~ 236 (1976)). As the photon quantum yield of luminol or isoluminol which is coupled to a ligand is reduced, it is preferred to use AHIE or ABEI.

The peroxidase enzyme (c) which is used in this invent ion includes peroxidase extracted from horseradish, for example, milk or leukocyte. Preferred is a peroxidase enzyme extracted from horseradish.

A suitable peroxidase enzyme (c) which is used for luminometric assay of this invention may either be coupled to a ligand such as an antigen, antibody, hapten, protein, abizin and biotin, or be free depending upon the type of assay. In the former case, a preferred ligand is an antigen. The ligand may directly coupled to the peroxidase enzyme or via a coupling agent. A preferred coupling agent is the well known N-(m-maleimidobenzoyloxy)succinimide (MBS).

A suitable oxidant (d) which is used in this invention may include hydrogen peroxide, sodium perborate and potassium perborate. Preferred is hydrogen peroxide.

To the assay of this invention, surfactant, bovine serum albumin, casein, gelatin, and the like may be added, if necessary.

The conditions for working of this invention will be described in detail.

The luminescent reaction of the present invention is preferably conducted under moderate conditions of temperature, ranging from 5 to 50°C., and pH, mor than 6; and most preferred to be conducted under conditions of temperature ranging from 10 to 50 °C, and pH, in the range of 6 to 11. Suitable buffer solutions which

may be used in the method of the present invention may be any type of buffers which are in the above-mentioned range. Among those buffer , preferred are phosphate buffer, glycine/NaOH buffer, Tris/HCL buffer, Tris/acetic acid buffer, carbonate buffer, barbital buffer, and borate buffer. The concentration of the forementioned buffer is preferred to be from 1 to 1000 mmol/l.

The concentrations of reaction substances which may be used in a luminescent reaction or luminometric assay excluding the substances to be assayed are generally kept constant. The concentration of a respective reaction substance used depends on the luminescent reaction, the object of the assay, method, and conditions. In general, suitable concentrations for the luminescent reaction or luminometric assay to be conducted are as follows: the phenolic derivative (a) of general formula (1) is preferred to be 10^4~ I (g/dl); the 2,3-dihydro-1,4-phthalazinedione or its derivative (b)
is preferred to be 100 nmol ~1 mol/liter, the peroxidase (c)
is preferred to be 1 ng ~5000 mg/liter, and the oxidant (d)
is preferred to be 100 nmol ~1 mol/liter.

Luminometric assays to which the present invention may be applied are now described in more detail. The light emitted may be quantified on a conventional luminometer (e. g., Luminesence Reader, made by Alka Co.).

As there are two types of immunoassay, homogeneous and heterogeneous, the luminometric assay of the present invention may me applied to moth of these. The homogeneous immunoassay and heterogeneous immunoassay wherein the luminometric assay of the present invention is applied will now be described.

In a homogeneous immunoassay, for example, either 2,3-dihydro-1,4-phthalazinedione or its derivative (b), or a peroxidase enzyme (c), both of which are preferably included in an assay kit of the present invention, is sealed into a liposome, and a ligand such as antigen, antibody, hapten, protein, avidin, and biotin is immobilized onto the surface of this liposome. An antigen-antibody reaction can then be carried out between the ligand in the sample and the immune-substance which is immobilized onto the surface of the liposome; consequently, the amount of the peroxidase or luminol which is derived from the liposome is quantified by conducting a luminometric assay involving a phenolic derivative (a) of a general formula (1) and an oxidant (d); and thus the ligand in the sample may be assayed.

The other type of immunoassay, heterogeneous immunoassay is a method,which is widely used for routin analysis in examination laboratories, is divided broadly into two categories comprising: (1) two-sit heterogeneous immunoassay; (2) competitive heterogeneous immunoassay. The luminometric assay of the present invention is applicable to either of both heterogeneous immunoassays.

In two-site heterogeneous immunoassay, a ligand such as an antigen, antibody, hapten, protein, avidin, and biotin is

immobilized on a carrier such as a test tube, glass beads, plastic beads, micro-particles, and the like, the sample is added thereto, then an antigen-antibody reaction is carried out therein. Secondly, after removing unreactant and adding enzyme-labelled ligand, an antigen-antibody reaction is carried out therein with the substances to be assayed in the sample which has been reacted and/or bound to the ligand which has been immobilized in the first step of the reaction.

Heterogeneous immunoassay employing two-site heterogeneous immunoassay may include a conventional enzyme immunoassay involving a solid phase antibody wherein an antiferritin-antibody is immobilized on a test tube made of polypropyrene, and antiferritin-antibody which is labelled by peroxidase. Enzyme activity is assayed by colorimetric assay involving a chromogen, 2,2'-azino-di(3-ethylbenzthiazoline-6-sulfonate)(ABTS), and the concentration of ferritin in the sample is measured by degree of this coloring (M.C. Revanet; Clin. Chem. vol.29, p.681 (1983)). By using instead of chromogen, a phenolic derivative of general formula (1), 2,3-dihydro-1,4-phthalazinedione or its derivative (b), and an oxidant (d), there is provided an enhanced luminescent reaction with more luminescence. The concentration of ferritin in the sample may be measured by quantifying the light emitted with accuracy and stability.

On the other hand, in competitive heterogeneous immunoassay wherein to the solid phase carrier such as test tube, glass beads, plastic beads, micro-particles, and the like, antibody is immobilized, then the substance to be assayed in a sample and enzyme labelled antigen of the substance to be assayed are allowed to carry out a competitive reaction therein. As the amount of the enzyme being bound to antibody being immobilezed on solid phase carrier is inversely proportional to the amount of the substance in the sample to be assayed, the amount of the substance to be assayed in the sample may be determined from a calibration curve which is prepared using a standard substance, in advance. In competitive heterogeneous immunoassay whose detail is shown by T.Arakawa et al (Anal. Biochem. vol.97 p.248(1974)), employing solid phase antigen wherein anticortisol antigen immobilized on Sepharose 4B and cortisol which are labelled by a peroxidase (c), the amount of the peroxidase being bound to the solid phase is assayed by a luminescent reaction using luminol and hydrogen peroxide, and thus cortisol in the sample is quantified. According to the present invention, a phenolic derivative (a) of general formula (1) is added to this luminescent reaction in order to enhance the luminescent

reaction, and maximize the sensitivity of the measurement by maximized light emission in order to quantify the cortisol in the sample with the sensitivity.

Accordingly, the present invention provides an assay kit for use in the enhanced luminescent or luminometric assay of the present invention. The assay kit preferably comprises each of;

(1) a phenolic derivative (a) of general formula (1)

(2) a 2,3-dihydro-1,4-phthalazinedione or its derivative (b)

(3) a peroxidase enzyme(c)

(4) an oxidant (d).

The assay kit may contain other additives as far as they do not inhibit their desired use.

Preferred combination of the assay kit will be each one of those substances mentioned above as preferred for use in the assay. In a most preferred embodiment of the assay kit at least one of the peroxidase enzyme (c) and 2,3-dihydro-1,4-phthalazinedione or its derivative (b) is coupled to an antibody to the substance to be assayed. The assay kit of this invention may comprise each of the abovementioned components (1) to (4) independently, or (1) is previously mixed to either one of (2), (3) and (4). Optionally the assay kit may also contain one or more standard solutions each containing a known amount of the substance to be assayed, and/or one or more of the preferred buffer solutions. Conveniently the assay kit may include a reaction vessel suitable for use in conjunction with the apparatus used for the determination of the light emission in the course of carrying out the assay. Also conveniently, a mixing device may be included in the assay kit, for use in mixing reactants.

Embodiments of the invention will now be described with reference to the following drawings and Examples.

In the drawings,

Figure 1 is a graph showing a luminescent pattern of luminol in the presence of (4-cyanomethylthio)phenol and is an illustration of the luminescent reaction embodying the present invention described in Example 1.

Figure 2 is a graph showing a comparison of the enhancing effects upon the chemiluminescence by (4-cyanomethylthio)phenol, 4-cyanomethylthio-2-chlorophenol, and paraiodophenol as described in Example 2.

Figure 3 is a graph showing a calibration curve of a chemiluminescent immunoassay indicating AFP.

Figure 4 is a graph showing correlation of the results of AFP assay by chemiluminescent immunoassay and chromometric immunoassay as described in Example 3.

Figure 5 is a graph showing a calibration curve of chemiluminescent immunoassay indicating T4.

Figure 6 is a graph showing a correlation of the results of the EIA assay indicating a chemiluminescent immunoassay embodying the present invention and chromometric immunoassay as described in Example 4.

Example 1

The present example illustrates that a chemiluminescent enhancer embodying the present invention promotes a chemiluminescent reaction among luminol, a coupled form of horseradish peroxidase, and hydrogen peroxide, and substantially increases the light emission compared with that achieved by the luminescent reaction in the absence of enhancer, and indeed in the presence of a known enhancer.

First, 0.02 mol/liter (hereinafter, referred to as M) of horseradish peroxidase (Grade I-C, manufactured by Toyobo Co., Ltd., Japan) was dissolved in phosphate buffer (pH 7.2). The concentration of the peroxidase in the solution was measured with an absorbance of 403 nm to be 0.12 mg/ml. The thus obtained solution was further diluted to 1/10000 by using the phosphate buffer.

Then, 0.18 g of luminol (manufactured by Tokyo Kasei Co., Ltd. Japan) and 0.1 g each of 4-(cyanomethylthio)phenol, 4-(cyanoethylthio)phenol, 4-(benzylthio)phenol, 4-(methylthio)phenol, 4-(morpholinomethylthio)phenol, 4-cyanomethylthio-2-fluorophenol, 4-cyanomethylthio-2-chlorophenol, 4-cyanomethylthio-2-bromophenol, 4-cyano-methylthio-2-iodophenol; and paraiodophenol as a control were respectively dissolved in 1 liter of Tris/hydrochloride buffer (0.1 M, pH 8.5) to obtain a stock solution.

As an oxidant, hydrogen peroxide was employed. 200 µl of 35 % hydrogen peroxide was dissolved in 1 liter of Tris/hydrochloride buffer (0.1 M, pH 8.5) to obtain a stock solution.

A luminescence reader (BLR-201 type, made by Aloca Co., Ltd.) was used for measuring a light intensity. The peroxidase solution (containing 1.2 ng of peroxidase) prepared as mentioned above was poured 100 µl at a time into test tubes made of glass having a dimension of 12 x 75 mm. The test tubes were set in sample holders of the luminescence reader. Then, 250 µl of luminol solution containing each enhancer was poured into the respective test tubes. Finally, the hydrogen peroxide solution were poured 250 µl at a time into the test tubes, thereby triggered the reaction (reaction temperature: 30 °C).

In the same way, a blank test, a test in the absence of the enhancer, and a comparative test using paraiodophenol as the enhancer were conducted. The results are shown in Table 1.

Table 1

	(1) POD 1.2ng	(2) POD 0ng	(1)/(2)
no enhancer	0.04	0.02	2
4-(cyanomethylthio)phenol	768.4	0.03	25613
4-(cyanoethylthio)phenol	7.3	0.01	730
4-(benzylthio)phenol	11.1	0.02	555
4-(methylthio)phenol	5.2	0.01	520
4-(morpholinomethylthio)phenol	18.6	0.02	930
4-cyanomethylthio-2-fluorophenol	31.1	0.02	1555
4-cyanomethylthio-2-chlorophenol	37.5	0.02	1875
4-cyanomethylthio-2-bromophenol	27.3	0.02	1365
4-cyano-methylthio-2-iodophenol	29.9	0.02	1495
paraiodophenol	27.9	0.02	1395

Remarks: POD: Horseradish Peroxidase Dimension; kilocount

The values (kilocount) listed in Table 1 were obtained by integrating a light emission for one minute from the time when the hydrogen peroxide was poured in. A luminescent pattern measured every three seconds when 4-(cyanomethylthio)phenol was added as an enhancer was as shown in Figure 1. This pattern becam constant about 15 seconds after the hydrogen peroxide was poured in.

Example 2

A comparison of optimum concentrations between the enhancers used in the present invention, such as 4-(cyanomethylthio)phenol, 4-cyanomethylthio-2-fluorophenol, 4-cyanomethylthio-2-chlorophenol, 4-cyanomethylthio-2-bromophenol and 4-cyanomethylthio-2-iodophenol, and the conventional enhancer such as paraiodophenol was made in this example.

First, 0.18 g of luminol (Tokyo Kasei Co.) was dissolved in 1 liter of Tris(hydrochloride buffer (0.1 M, pH 8.5). Then, 4-(cyanomethylthio)phenol, 4-cyanomethylthio-2-fluorophenol, 4-cyanomethylthio-2-chlorophenol, 4-cyanomethylthio-2-bromophenol, 4-cyanomethylthio-2-iodophenol, and paraiodophenol were respectively dissolved in the thus obtained solution so that each concentration became 0.1 g/dl, 0.05 g/dl, 0.01 g/dl, 0.005 g/dl, and 0.001 g/dl, thereby obtaining stock solutions.

The luminescense reader (Alkca Co.; BLR-201) was used for measuring light intensity. The peroxidase solution (containing 1.2 ng of peroxidase) prepared as mentioned above was poured 100 µl at a time into test tubes made of glass having a dimension of 12 x 75 mm. The test tubes were set in sample holders of the luminescense reader. Then, 250 µl of luminol solution containing the enhancer of each concentration was poured into the respective test tubes. Finally, the hydrogen peroxide solution was poured 250 µl at a time into the test tubes, thereby triggered the reaction (reaction temperature: 30 °C).

The results are shown in Table 2.

The values (kilocount) listed in Table 2 were obtained by integrating a light emission for one minute from the time when the hydrogen peroxide was poured in. Figure 2 shows a relationship between the respective con-

centrations of the enhancers and the blank values of the light intensity. From these results, it was found that the 4-(cyanomethylthio)phenol used in the present invention exhibited more excellent performance (the light emission and the ratio of the light emission to the blank value) than the paraiodophenol of the prior art, even when the concentration of the 4-(cyanomethylthio)phenol was 1/10 of that of the paraiodophenol. Moreover, 4-cyanomethylthio-2-fluorophenol, 4-cyanomethylthio-2-chlorophenol, 4-cyanomethylthio-2-bromophenol, and 4-cyanomethylthio-2-iodophenol exhibited an excellent performance as compared with paraiodophenol, even when the concentrations of the above phenols used in the present invention were the same as that of the paraiodophenol.

Table 2

Enhancer	Sample	g/dl				
		0.1	0.05	0.01	0.005	0.001
4-(cyanomethyl thio)phenol	①	107.5	307.3	768.4	386.2	4.2
	②	0.02	0.02	0.03	0.02	0.01
	①／②	5375	15365	25613	19310	420
4-cyanomethyl thio-2-fluorophenol	①	297.1	429.0	31.1	4.5	0.8
	②	0.02	0.02	0.02	0.01	0.01
	①／②	14855	21450	1555	450	80
4-cyanomethyl thio-2-chlorophenol	①	338.4	455.3	37.5	6.3	1.3
	②	0.02	0.02	0.02	0.01	0.01
	①／②	16920	22765	1875	630	130
4-cyanomethyl thio-2-bromophenol	①	316.2	396.1	27.3	5.1	0.9
	②	0.02	0.02	0.02	0.01	0.01
	①／②	15810	19805	1365	510	90
4-cyanomethyl thio-2-iodophenol	①	351.2	439.6	29.9	6.9	1.5
	②	0.02	0.02	0.02	0.01	0.01
	①／②	17560	21980	1495	690	150
paraiodophenol	①	85.6	278.1	27.9	7.2	0.9
	②	0.01	0.02	0.02	0.01	0.01
	①／②	8560	13905	1395	720	90

Remarks: Dimension; kilocount

①; light emission POD 1.2 ng

②; light emission POD 0 ng (blank)

①／②: the ratio of the light emission to the blank value

Example 3

This example describes measurement of α- phetoprotein (hereinafter, referred to as AFP) by two-site chemiluminescent immunoassay in which 4-(cyanomethylthio)phenol is used as a luminescent enhancer, and a solid phase antibody, a peroxidase labelled antibody, luminol, and hydrogen peroxide are combined.

(1) Immobilization of anti-AFP antibody on test tubes:

An anti-AFP antibody (derived from a rabbit; manufactured by Dako Co., Ltd.) was dissolved in carbonate buffer (0.02 M, pH 9.5) so as to be 100 μ g/ml. The carbonate buffer containing the anti-AFP antibody was poured 500 μl at a time into test tubes made of polystyrene having a dimension of 12 x 75 mm (manufactured by Nunk Co., Ltd.) and allowed to react for 48 hours at 4 °C, thereby immobilizing the antibodies on inner walls of the test tubes. After the reaction was completed, liquid in the test tubes was removed by using an aspirator. The test tubes were washed with 1 ml of a physiological saline three times. Then, 1 ml of phosphate buffer (0.02 M, pH 7.2) containing 1 % bovine serum albumin (hereinafter, referred to as BSA) was added to each test tube, and kept at 4°C until they were used.

(2) Preparation of enzyme labelled anti-AFP antibody:

Peroxidase labelled anti-AFP antibody was prepared from horseradish peroxidase (Toyobo Co.; I-C,) and anti-AFP antibody (derived from rabbit, manufactured by Dako Co., Ltd.) by the periodate oxidation method (Nakane et al; J. Histochem. Cytochem., vol.22, p.1,084 (1974)).

(3) Measurement of AFP in the blood:

(i) Enzyme labelled antibody solution;
The peroxidase labelled anti-AFP antibody obtained in (2) was diluted with phosphate buffer (0.02 M, pH 7.2) containing 1 % BSA so as to be at a concentration required for the measurement.
(ii) Standard solution
Purified human AFP was diluted with phosphate buffer (0.02 M, pH 7.2) containing 1 % BSA so that each concentration became 20 ng/ml, 80 ng/ml, 320 ng/ml, and 640 ng/ml.
(iii) Measurement method
An immersed solution in the test tubes in which antibodies were immobilized on the inside walls thereof prepared in (1) was removed by using an aspirator. The test tubes were washed with 1 ml of physiological saline once. Then, 30 μl of standard solution or specimen (serum or plasma) was sampled in the washed test tubes. 500 μl of phosphate buffer (0.02 M, pH 7.2) containing 1 % BSA was added to the standard solution or specimen in the test tubes and thoroughly stirred, followed by incubation for 30 minutes at 37 °C.
After the reaction was completed, the reaction solution was removed by using an aspirator. Then, the test tubes were washed with 1 ml of physiological saline again. The same procedure was repeated three times to remove various serum components in the specimen.
Next, 500 μl of enzyme labelled anti-AFP antibody solution prepared in (i) was poured into the test tubes and thoroughly stirred, followed by incubation for 30 minutes at 37 °C. After the reaction was completed, the reaction solution was removed by using an aspirator. The test tubes were washed with 1 ml of physiological saline again. This procedure was repeated three times to remove unreacted enzyme labelled anti-AFP antibody.
The test tubes washed in the above were set in the sample holders of the luminescense reader. Then, 250 μl of luminol solution containing 4-(cyanomethylthio)phenol and 250 μl of hydrogen peroxide prepared in Example 1 were poured into the respective test tubes, whereby the chemiluminescent reaction was conducted. The measurement values were obtained by integrating a light emission for one minute.
The light emission at each concentration of the standard solution when the standard solution was used as a specimen is shown in Table 3.

Table 3

Standard AFP (ng/ml)	0	20	80	320	640
Light emission (kilocount)	0.11	31.9	124.7	463.2	821.2

The AFP concentration was read from a calibration curve (Figure 3) obtained by plotting a light emission at each concentration of the standard solution.

Figure 4 shows a relationship between the AFP concentration obtained by measuring the specimen according to the method of this example and the AFP concentration obtained by measuring the specimen in accordance with chromometric EIA (Enzyme Immunoassay) using the peroxidase labelled anti-AFP antibody.

Example 4

This example describes quantification of thyroxine (T4) by a chemiluminescent immunoassay in which 4-(cyanomethylthio)phenol is used as a luminescent enhancer, and a solid phase antibody, a peroxidase labelled antigen, luminol, and hydrogen peroxide are combined.

(1) Immobilization of anti-T4 antibody on the test tubes:

Anti-T4 antibody (derived from rabbit, manufactured by Kallestad Co., Ltd.) was immobilized on the inner walls of the test tubes in the same way as in Example 3.

(2) Preparation of enzyme labelled antigen (T4):

Peroxidase labelled T4 was prepared from horseradish peroxidase (Toyobo Co.; I-C) and T4 (manufactured by Calbiochemistry Co., Ltd.) by the glutaraldehyde method (Abrameas; Immunochemistry, vol.6, p.43 (1969)).

(i) Enzyme labelled antigen solution The peroxidase labelled antigen (T4) obtained in (2) was diluted with barbital buffer (0.1 M, pH 8.6) containing 1 % BSA and 0.04 % 8-anilino-1-naphthalenesulfonic acid ammonium so as to be at a concentration required for the measurement.

(ii) Standard solution

T4 (Calbiochemistry Co.) was added to T3 and T4-free serum obtained by treating human serum with activated carbon so that the concentration became 2.0 μg/dl, 6.0 μg/dl, 12.0 μg/dl, and 24.0 μg/dl in accordance with the method of Miyai et al., (Miyai et al; Endocrinol Japan, vol.27, p.375 (1980)).

(iii) Measurement method

The test tubes were washed in the same way as in Example

3. Then, 20 μl of standard solution or specimen (serum or plasma) was sampled in the washed test tubes. 500 μl of enzyme labelled antigen solution was added to the standard solution or specimen in the test tubes and thoroughly stirred, followed by incubation for 30 minutes at 37 °C.

After the reaction was completed, the reaction solution was removed by using an aspirator. Then, the test tubes were washed with 1 ml of physiological saline again. The same procedure was repeated three times to remove various serum components in the specimen and unreacted enzyme labelled antigen (T4).

The test tubes washed in the above were set in the sample holders of the luminescense reader. The chemiluminescent reaction was conducted in the same way as in Example 3. The measurement values were obtained by integrating a light emission for one minute.

The light emission at each concentration of the standard solution when the standard solution was used as a specimen is shown in Table 4.

Table 4

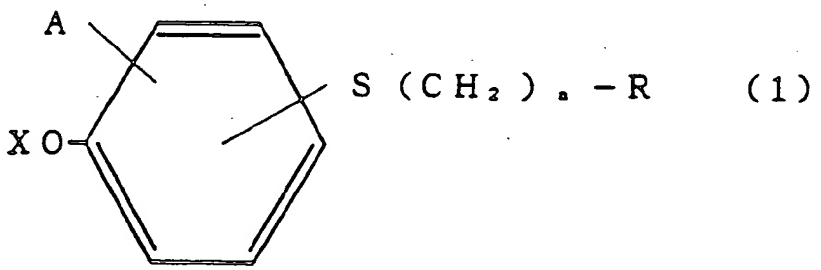
Standard T4(μg/dl)	0	20	80	320	640
Light emission (kilocount)	628.4	421.2	315.3	208.4	97.3

The T4 concentration in the specimen (serum or plasma) was read from a calibration curve (Figure 5) obtained by plotting light emission at each concentration of the standard solution.

Figure 6 shows a relationship between the T4 concentration obtained by measuring the specimen according to the method of this example and the T4 concentration obtained by measuring the specimen in accordance with chromometric EIA using the peroxidase labelled antigen (T4).

Claims

1. A method of enhancing a luminescent reaction involving 2,3-dihydro-1,4-phthalazinedione or its derivative, a peroxidase enzyme and an oxidant, which method comprises conducting the reaction in the presence of an enhancer which is a phenol derivative, characterised in that phenolic derivative has the general formula (1)



(1)

wherein n represents an integer of 1 to 5; R is a hydrogen atom or a cyano, morpholino, carboxylic acid, C_{2-7} alkoxycarbonyl, metallic carboxylate, aldehyde or allyl group; or is a phenyl group optionally substituted by a halogen atom; A is a hydrogen or halogen atom; X is hydrogen or alkaline metal; and optionally, OX and $S(CH_2)_n$ -R are in an ortho or para position relative to one another.

2. A luminometric assay which involves the enhanced luminescent reaction according to claim 1.

3. A luminometric assay according to claim 2 in which at least one of the peroxidase and the 2,3-dihydro-1,4-phthalazinedione or its derivative is or are employed as an assay reagent.

4. A luminometric assay according to claim 2 comprising detecting the presence of a peroxidase.

5. A luminometric assay according to claim 2 comprising detecting the presence of a 2,3-dihydro-1,4-phthalazinedion or its derivative.

6. A luminometric assay according to any one of claims 2 to 5, wherein either one of 2,3-dihydro-1,4-phthalazinedione or its derivative, or peroxidase are in ligand coupled form.

7. A luminometric assay according to claim 6, wherein the ligand is an antigen, hapten, protein A, avidin or biotin.

8. A luminometric assay according to any one of claims 2 to 7, wherein the 2,3-dihydro-1,4-phthalazinedione or its derivative is luminol, N-aminohexyl-N-ethylisoluminol, or N-aminobutyl-N-ethyisoluminol.

9. A luminometric assay according to any one of claims 2 to 8, wherein the phenolic derivative is (4-benzylthio)phenol, (4-methylthio)phenol, (4-cyanomethylthio)phenol, (4-morpholinomethylthio)phenol, 4-cyanomethylthio-2-fluorophenol, 4-cyanomethylthio-2-chlorophenol, 4-cyanomethylthio-2-bromophenol, or 4-cyanomethylthio-2-iodophenol.

10. A luminometric assay according to claim 9, wherein the phenolic derivative is (4-cyanomethylthio)phenol.

11. A luminometric assay according to any one of claims 2 to 10, wherein the peroxidase enzyme is a horseradish peroxidase.

12. A luminometric assay according to any one of claims 2 to 11, wherein the oxidant is hydrogen peroxide, or perborate.

13. A kit for use in a luminescent or luminometric assay comprising a phenolic derivative of the general formula (1), defined in claim 1, an oxidant, and at least one of a 2,3-dihydro-1,4-phthalazinedione or its derivative and a peroxidase enzyme.

14. A kit according to claim 13, comprising each of the said phenolic derivative, oxidant, 2,3-dihydro-1,4-phthalazinedione or its derivative and peroxidase enzyme.

15. A kit according to claim 13 or 14, wherein either one of 2,3-dihydro-1,4-phthalazinedione or its derivative, or peroxidase is coupled with a ligand.

16. A kit according to claim 14, wherein the ligand is an antigen, antibody, hapten, protein A, avidin, or biotin.

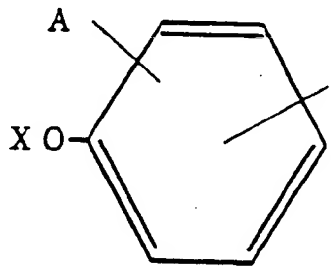
17. A kit according to any one of claims 13 to 16, wherein the 2,3-dihydro-1,4-phthalazinedione or its derivative is luminol, N-aminohexyl-N-ethylisoluminol, or N-aminobutyl-N-ethylisoluminol.

18. A kit according to any one of claims 13 to 14, wherein the phenolic derivative is (4-cyanomethylthio)phenol.

19. A kit according to any one of claims 13 to 18, wherein the peroxidase enzyme is horseradish peroxidase.

20. A kit according to any one of claims 13 to 19, wherein the oxidant is hydrogen peroxide, or perborate.

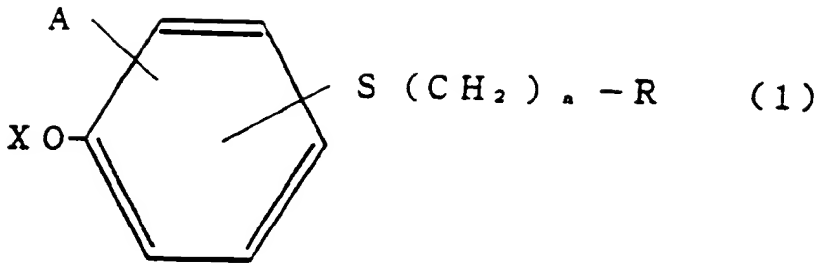
21. A luminescent which comprises carrying out a luminescent reaction involving 2,3-dihydro-1,4-phthalazinedione or its derivative, a peroxidase enzyme, an oxidant, and a phenolic derivative illustrated below by the general formula (1)



(1)

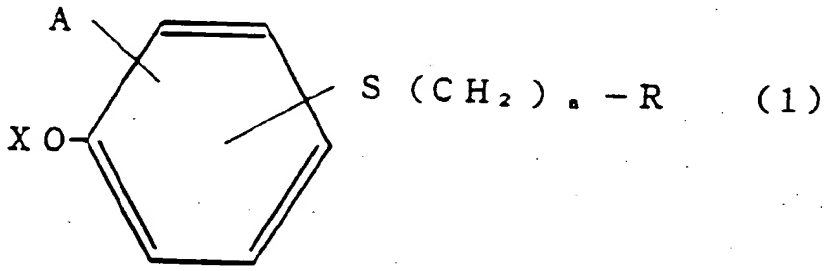
wherein n represents an integer of 1 to 5; R is a hydrogen atom, a cyano, morpholino, carboxylic acid, C_{2-7} alkoxycarbonyl, metallic carboxylate, amido, aldehyde, or allyl group, or is a phenyl group optionally substituted by a halogen atom; A is a hydrogen atom or a halogen atom; X is hydrogen or alkaline metal; and optionally, OX and S $(CH_2)_n$ -R are in an ortho or para position relative to one another.

22. A luminescent which is the reaction product of a luminescent reaction involving 2,3-dihydro-1,4-phthalazinedione or its derivative, a peroxidase enzyme, an oxidant, and a phenolic derivative having the general formula (1)



wherein n represents an integer of 1 to 5; R is a hydrogen atom or a cyano, morpholino, carboxylic acid, C_{2-7} alkoxycarbonyl, metallic carboxylate, amido, aldehyde, or allyl group; or is a phenyl group optionally substituted by a halogen atom; A is a hydrogen atom or a halogen atom; X is a hydrogen or alkaline metal; and optionally, OX and S $(CH_2)_n$ -R are in an ortho or para position relative to one another.

23. Use of a phenol derivative for enhancement of a luminescent reaction involving 2,3-dihydro-1,4-phthalazinedione or its derivative, a peroxidase enzyme and an oxidant, characterised in that the phenolic derivative has the general formula (1)



wherein n represents an integer of 1 to 5; R is a hydrogen atom or a cyano, morpholino, carboxylic acid, C_{2-7} alkoxycarbonyl, metallic carboxylate, amido, aldehyde, or allyl group; or is a phenyl group optionally substituted by a halogen atom; A is a hydrogen atom or a halogen atom; X is a hydrogen or alkaline metal; and optionally, OX and S $(CH_2)_n$ -R are in an ortho or para position relative to one another.

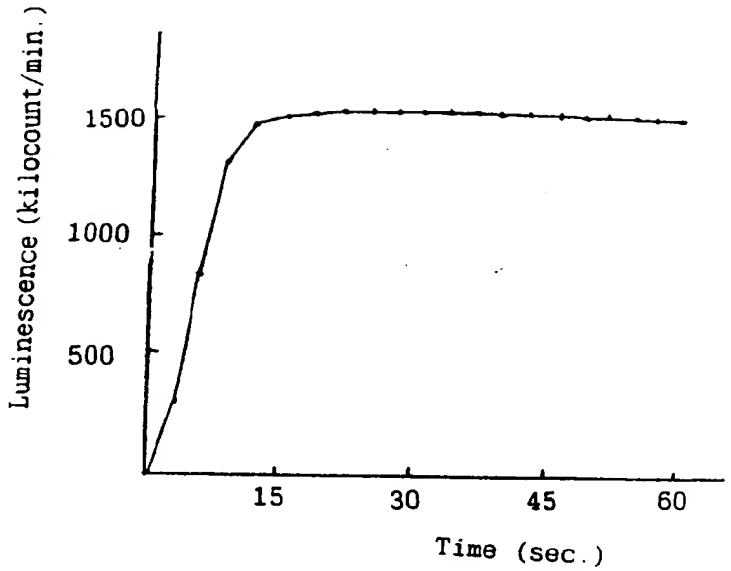


Fig. 1 Luminescent pattern of luminol added (4-cyanomethylthio)phenol

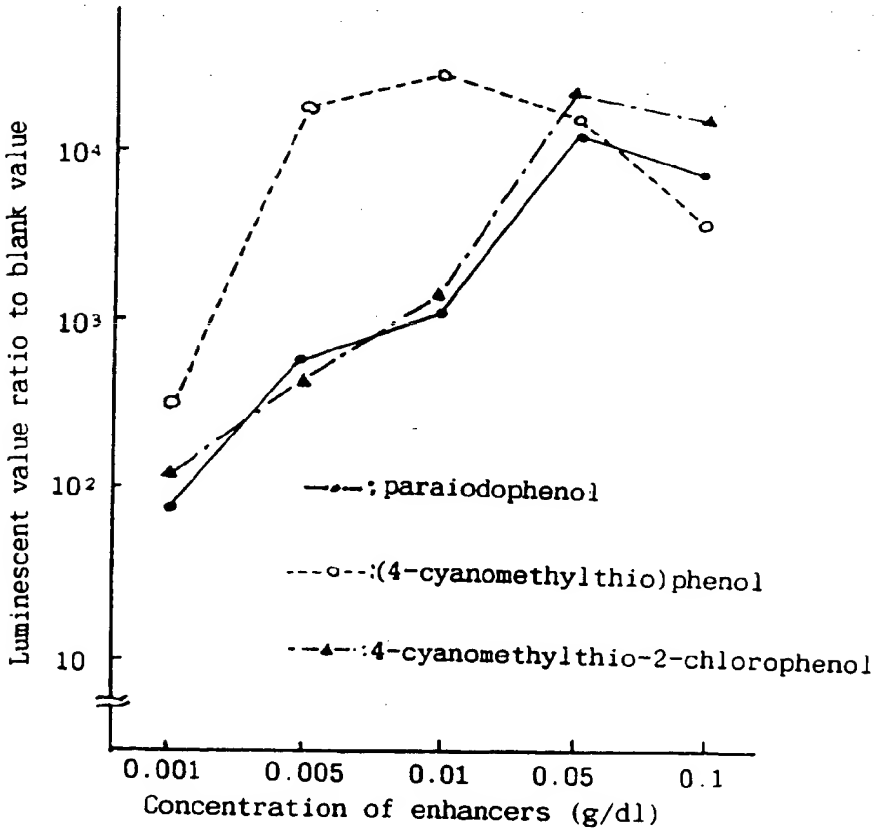


Fig. 2 Comparison of enhancing effects

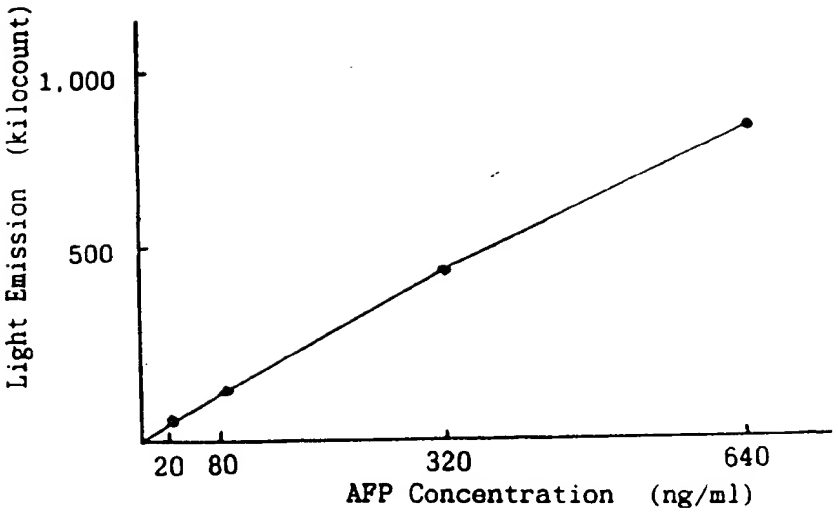


Fig. 3 Calibration curve of chemiluminecent immunoassay indicating AFP.

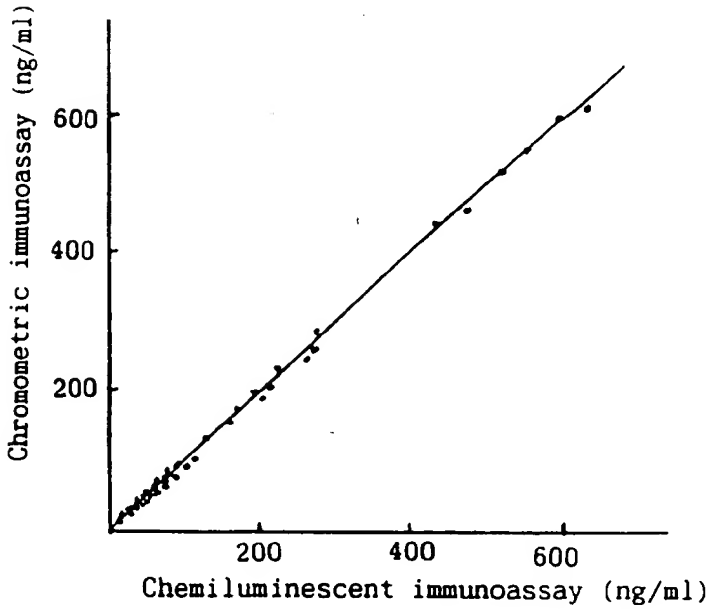


Fig. 4 Correlation of AFP assay by chemiluminescent and chromometric immunoassay

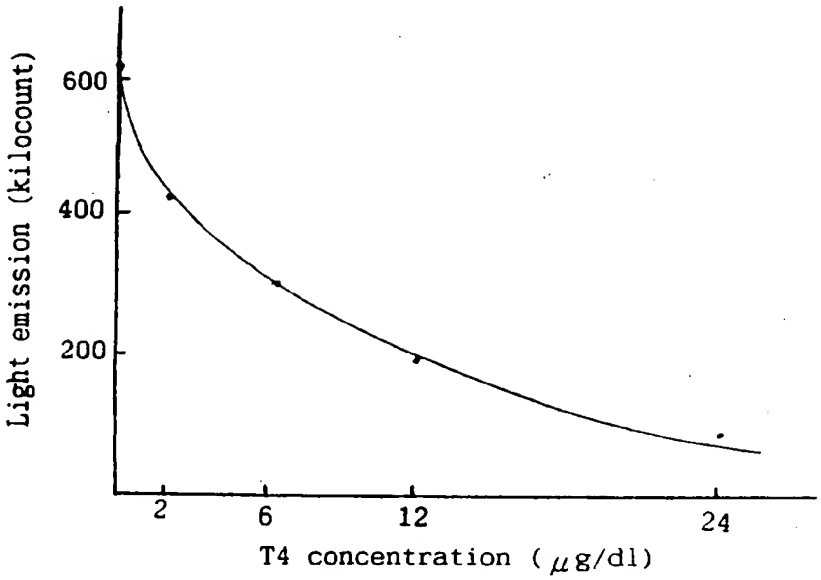


Fig. 5 Calibration curve of chemiluminescent immnoassay indicating T4.

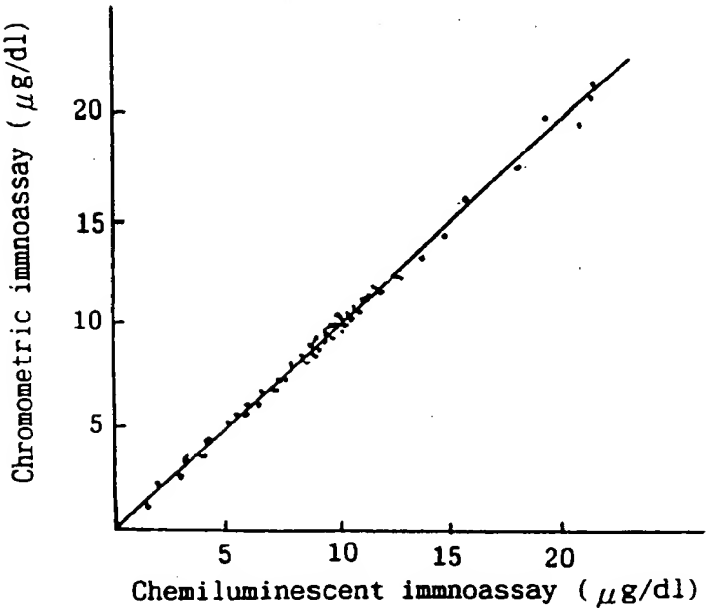


Fig. 6 Correlation of EIA assay indicating chemiluminescent and chromometric immunoassay

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 92 30 2414

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A	EP-A-0 116 454 (NATIONAL RESEARCH DEVELOPMENT CORPORATION) * the whole document * &US-A-4 598 044, category D	1-23	C12Q1/28 G01N33/58
A	METHODS IN ENZYMOLOGY vol. 133, 1986, NEW YORK, US pages 331 - 353; GARY H. THORPE AND LARRY J. KRICKA: 'Enhanced Chemiluminescent Reactions Catalyzed by Horseradish Peroxidase' * page 335, line 5 - line 21 * * page 336, line 18 - page 340; table V * * page 342; table VII *	1-23	
D,A	CLINICAL CHEMISTRY. vol. 31, no. 8, August 1985, WINSTON US pages 1335 - 1341; G.H. THORPE, L.J. KRICKA, S.B. MOSELEY, T.P. WHITEHEAD: 'Phenols as Enhancers of the Chemiluminescent Horseradish Peroxidase-Luminol-Hydrogen Peroxide Reaction: Application in Luminescence-Monitored Enzyme Immunoassays' * the whole document *	1-23	TECHNICAL FIELDS SEARCHED (Int. Cl.5) C12Q G01N

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	03 JUNE 1992	DöPFER, K.P.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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DARBY & DARBY P.C.
Attn. Schaffer , Robert
805 Third Avenue
New York, N.Y. 10022-7513
UNITED STATES OF AMERICA

Date: 26/02/2002

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When? Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How? Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped),whether

(i) the claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international application is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.